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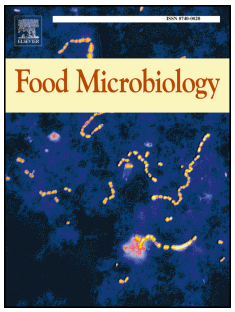

Microbial diversity and dynamics of Spanish-style green table-olive fermentations in large manufacturing companies through culture-dependent techniques

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Microbial diversity and dynamics of Spanish-style green table-olive fermentations in large manufacturing companies through culture-dependent techniques.

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Abstract

We have studied the microbiota associated to Spanish-style green olive fermentations, attending to its dynamics along the time. Twenty 10-tonne fermenters were selected from two large table-olive manufacturing companies in southern Spain. While culture-dependent methodology was used to isolate the microorganisms, molecular methods were used to identify the isolates. A total of 1070 isolates were obtained, resulting in 929 bacterial and 141 yeast isolates. Thirty seven different bacterial species were isolated, belonging to 18 different genera, while 12 yeast species were isolated, belonging to 7 distinct genera. This fermentation was dominated by the species *Lactobacillus pentosus,* while its accessory microbiota was variable and depended on the fermentation stage and the actual fermentation yard ("*patio*"). It was noticeable the abundance of lactic acid bacteria isolates, belonging to 16 different species. Twenty bacterial species were isolated for the first time from Spanish-style green olive fermentations, while 17 had not been described before in any table olive preparation. The genera *Brachybacterium*, *Paenibacillus, Sporolactobacillus, Paracoccus* and *Yersinia* had not been cited before from any table olive preparation. *Saccharomyces cerevisiae* and *Candida thaimueangensis* appeared to dominate the yeast microbiota. *Candida butyri/asseri* and *Rhodotorula mucilaginosa* had not been described before from Spanish-style preparations, while *Saturnispora mendoncae* was isolated for the first time from any table olive preparation. Biodiversity was analysed through different alpha and beta indexes which showed the evolution of the microbiota over time.

Keywords: olive fermentation, biodiversity, microbiota, lactic acid bacteria, yeast, *Lactobacillus pentosus*.

1. Introduction

Table olives account for the largest volume of fermented vegetables in Western countries, especially in Mediterranean countries (Garrido-Fernández et al., 1997). World production reached an average of 2.3 million tons per year in the period 2006-2012 (IOOC, 2012). Although table olives can be prepared for consumption in many different ways, Spanish-style preparation of green olives is one of the three most commercially important worldwide, along with natural black olives and oxidised black olives (Garrido Fernández et al., 1995; Sánchez et al., 2006; Rejano et al., 2010), representing 60% of the world production (Botta and Cocolin, 2012). Spanish-style preparation is characterised by the initial alkali treatment (1.8-3.5 % [w/v] NaOH) of the green fruits, which removes bitterness and allows the subsequent growth of lactic acid bacteria (LAB) through the neutralisation and washing of inhibitory phenolic compounds (Rejano et al., 2010). Once removed the alkali, fruits are washed once or twice with water and finally covered with brine (10-12 % [w/v] NaCl). In this brine a spontaneous fermentation takes place in which at least three different stages have been identified (Garrido-Fernández et al., 1995). During the first stage, usually lasting 3-10 days, fermentation is conducted by the indigenous alkali-tolerant microbiota which contaminates the fruits as well as the environment (de Castro et al., 2002). This microbiota is responsible for lowering the initial high pH (10-11) to values close to 6-7, more appropriate for the growth of LAB, which are also present as contaminants (Sánchez et al., 2001). As soon as LAB take over and grow exponentially, during what it is considered the second stage in this fermentation, pH value drops as a result of their metabolism. Sugars are converted into lactic acid, as the major product, as a result of a mainly homolactic fermentation. This is carried out mostly by strains of the species *Lactobacillus pentosus* (de Castro et al., 2002; Rejano et al., 2010; Ruiz-Barba and Jiménez-Díaz, 2012), although in the past this role was attributed to strains of *Lactobacillus plantarum* (Ruiz-Barba et al., 1994; Garrido-Fernández et al., 1995; Rejano et al., 2010) as a consequence of previous phenotypic criteria for the classification of species into what it is known as the "*L. plantarum* group", before molecular criteria were applied (Torriani et al., 2001). At the end of the second stage, typically 10 to 15-day long, pH value is about 4.5 and most sugars have been utilised (Montaño et al., 1993; Garrido-Fernández et al., 1995). During the final, third stage of the fermentation all fermentative substrates are exhausted and LAB population declines

steadily. Values of pH below 4.0 and free acidity of 0.7-1.2 %, mainly as lactic acid, are considered indicative of a good fermentation. These conditions, combined with a NaCl concentration which is at this stage usually raised to 7-8 %, should guarantee the long-term preservation of the final product.

Up to date, few comprehensive studies have been carried out on the microbiota of table olive fermentations, especially if we consider modern taxonomic criteria and molecular techniques (Ercolini et al., 2006; Botta and Cocolin, 2012; Cocolin et al., 2013). The aim of this study is to update the knowledge we have about the microbial diversity, in terms of both bacteria and yeast, which is inherent to the Spanish-style fermentation of green olives in large scale table-olive manufacturing companies. For this, we have used culture-dependent techniques for the isolation of the different microorganisms as well as molecular techniques to obtain as precise identifications as possible. We have selected two different large-scale table-olive fermentation yards (known in Spanish as "*patios*"), belonging to two large table-olive manufacturing companies in the province of Seville, southern Spain. In this province, up to 63% of the Spanish national production is concentrated (season 2012/2013; AAO, 2013), so that data obtained should be quite relevant. Actually, this table olive preparation is also known as "Sevillian-style" (Rejano et al., 2010). Finally, our goal is to obtain not only a picture of the microbial diversity along the time of this food fermentation but also get a well characterised collection of microorganisms to be used in the future as a comprehensive bank of wild-type strains for diverse biotechnological uses.

2. Materials and Methods

2.1. Origin of the samples and sampling strategy.

Samples of Spanish-style green-olive fermenting brines were taken during the 2010-2011 season from two large (4,000 to 8,000 tonnes of olives handled per season) table-olive manufacturing companies in the province of Sevilla, south-western Spain. These companies are located *ca.* 35 Km apart from each other. At each company, fermentation was followed in ten fermenters. These were of a total capacity of 10 tonnes of olives and 5,500-6,000 litres of brine, made in polyester and glass fibre. They were all located outdoor, buried in the ground of what it is traditionally called in Spain a "*patio*". The traditional Spanish-style procedure to prepare green olives was followed

(Rejano et al., 2010). Briefly, green olives were treated with a solution of NaOH (2-2.5 % [w/v]) with the addition, only in the case of *patio* #1, of NaCl (15.3 g/L) and $CaCl_2$ (0.83 g/L), for 8-10 hours; the olives were then washed with water to remove the excess of alkali and finally covered with brine (10-11 % [w/v] NaCl). Again, only in the case of *patio* #1, brine contained 1.87 g/L $CaCl_2$. At this point, treated olives plus brine are used to fill up the 10-tonne fermenters located in the *patios*. Only in *patio* #1, brines were acidified by the addition of 25 litres of food-grade HCl. After 1-2 moths of fermentation, in both *patios ca.* 500 L of the fermenting brine taken from the bottom of the fermenters, and containing olive debris and more alkaline conditions, were discarded. The fermenters were then refilled with fresh brine containing lactic acid and HCl (usually 5 L each), being this a common practice in large table-olive manufacturing companies to avoid spoilage. Olives were all of the Manzanilla variety and no starter culture was used. Fermentations were set up during September 2010 and three consecutive 50-ml samples were taken from each fermenter at approximately monthly intervals, in coincidence with the initial, middle and final stages of green olives fermentation. As the harvesting of the fruits as well as the processing capacity of these industries had an obvious daily limitation, only a limited number of fermentations could be set up daily. Therefore, at each of the three sampling dates, brine samples collected from the fermenters at each *patio* felled into a range of time after brining. More specifically, fermentation had taken place for 1 to 14 (first two weeks), 35 to 48 (5th to 7th week), and 69 to 72 (10th to 12th week) days after brining, for sampling points #1, 2 and 3, respectively. Samples were added glycerol so that final concentration was 20 % (v/v) and stored at -80ºC until use.

2.2. Isolation of microorganisms.

Aliquots of samples stored at -80ºC were defrost at room temperature, serially diluted in 0.1 % (w/v) peptone water and extended in duplicates onto agar plates of culture media. Five different culture media were used in this study: Brain Heart Infusion (BHI; Biokar Diagnostics, Beauvais, France) supplemented with 0.05% L-cysteine (AppliChem, Darmstadt, Germany); de Man-Rogosa-Sharpe (MRS; Biokar Diagnostics) supplemented with 0.02 g/L bromophenol blue (AppliChem) and L-cysteine (MRS-BPB; Lee and Lee, 2008); Reinforced Clostridial Medium (RCM; Biokar Diagnostics); and MacConkey Broth Purple (Biokar Diagnostics). Seeded plates were incubated anaerobically at 30 ºC for three days, except for RCM, when seven-day

incubations were used. For anaerobic incubations we used a DG250 Anaerobic Workstation (Don Whitley Scientific Ltd., Shipley, West Yorkshire, UK), with a gas mixture consisting of 10% H_2-10% CO_2-80% N_2. Glucose-Yeast Extract Agar supplemented with oxytetracycline (0.1 g/L) (OGYE; Mossel et al., 1962) was incubated aerobically at 30 °C for 2 days. Agar was added to the broth media at 1.5 % (w/v). Prior to spreading onto RCM agar plates, samples were pasteurised at 75 ºC for 15 min in a water bath. For further studies, a single colony of each different morphotype identified in each culture medium at every sampling point was selected from plates with low counts, purified by repeated subculturing and observed under a phase-contrast microscope (Olympus Optical Co., Tokyo, Japan) to distinguish its cell morphology. For long-term storage, purified isolates were preserved at -80 ºC in their culture medium containing glycerol (20% v/v). All isolates were subjected to genotyping as described below.

2.3. Molecular identification techniques.

Total DNA was extracted directly from colonies by the rapid chloroform method described by Ruiz-Barba et al. (2005). The same DNA extraction, preserved at 4 ºC, was used for all subsequent molecular techniques. Primers used in this study are described in Table 1.

2.3.1. Genotyping by Randomly Amplified Polymorphic DNA (RAPD).

Microbial isolates were grouped by their cell morphology before strain typification by the RAPD fingerprinting technique. Genotyping was carried out by RAPD using the primer OPL5 as described by Maldonado-Barragán et al. (2013). In the case of coccus-shaped bacteria, primer ISS1rev was used instead. The resulting RAPD profiles were normalized and analyzed for clustering with the Bionumeric 7.0 software package (Applied Maths, Sint-Martens-Latem, Belgium). Only bands representing amplicons between 150 and 5000 bp in size were included in the analysis. Similarity dendrograms were constructed by the UPGMA clustering method, using the band-based Dice similarity coefficient. Similarity coefficient ≥0.80 was considered as a cut-off value for isolates belonging to the same strain. A representative isolate of each RAPD profile was selected for further characterization.

2.3.2. 16S rDNA sequence analysis of bacterial isolates.

Representative bacterial isolates were identified to the genus level and/or to the species level by PCR sequencing of a *ca*. 500-bp fragment of the 16S rDNA gene, using the primer pair plb16/mlb16. PCR conditions were as described by Delgado et al. (2008). Briefly: initial denaturation at 96°C for 30 s, followed by 30 cycles of denaturation at 96 ºC for 30 s, annealing at 50°C for 30 s, and polymerisation at 72°C for 45 s, plus a final polymerisation step at 72°C for 4 min. MyTaq DNA polymerase (Bioline, London, UK) was used according to the manufacturer instructions. The resulting amplicons were purified using a Nucleospin Extract II kit (Macherey-Nagel, Düren, Germany) and sequenced at Newbiotechnic S.A. (Bollullos de la Mitación, Spain). The resulting sequences were used to search for similarities in the relevant databanks using the Nucleotide BLAST utility at the NCBI web page (http://blast.ncbi.nlm.nih.gov/Blast.cgi) by limiting this search to type strains (*"Entrez query" option*). The identities of the representative isolates were determined on the basis of the highest scores (typically ≥98%).

2.3.3. PCR amplification with species-specific primers.

Species-specific PCRs were performed for further discrimination when the results of 16S rDNA sequence analysis were not enough to identify species belonging to some bacterial groups. Species belonging to the *L. plantarum* group, i.e. *L. plantarum*, *L. pentosus* and *Lactobacillus paraplantarum*, were distinguished using a multiplex PCR assay with the *recA* gene-based primers paraF, pentF, planF and pREV as described by Torriani *et al.* (2001). Species belonging to the *Lactobacillus casei* group, i.e. *L. casei*, *Lactobacillus paracasei* and *Lactobacillus rhamnosus*, were distinguished using a multiplex PCR assay with the *tuf* gene-based primers CAS, PAR, RHA and CPR as described by Ventura *et al.* (2003).

2.3.4. 26S rDNA sequence analysis of yeast isolates.

Representative yeast isolates were identified to the genus level and/or to the species level by PCR sequencing of the D1/D2 domain of the 26S *rDNA* gene (Kurtzman and Robnett, 1998). For this purpose, PCR amplification of the 26S rDNA gene using the universal primers NL1 and NL4 was performed as described by Kurtzman and Robnett (1998). The resulting amplicons were purified, sequenced and analysed according to the criteria for the differentiation of yeast species defined by

Kurtzman and Robnett (1998), who considered a similarity higher than 99% to assign an isolate to a yeast species after doing a BLAST search in the relevant data banks.

2.4. Physico-chemical analyses.

Titratable acidity, expressed as g/L lactic acid, combined acidity, expressed as Eq/L NaOH, and pH were measured using a Metrohm 670 Titroprocessor (Herisau, Switzerland). Salt concentration was determined by titration with $AgNO_3$ and expressed as % (w/v) NaCl.

2.5. Statistical analyses.

Total counts of microorganisms were expressed as the mean values of colony forming units (CFU) per milliliter of brine based on duplicate analyses made for each sample, including the standard deviation (SD) of the mean. The resulting values were transformed to logarithmic values before statistical analyses were performed. U Mann-Whitney tests were applied to determine statistically significant differences between the microbial counts in both *patios* at each sampling point and for each culture media used. The fermentation-time effect on averaged microbial counts recovered from each culture media in both *patios* was tested using Friedman tests. These analyses were performed using the SPSS 21.0 statistical software (SPSS Inc., Chicago, USA).

2.6. Biodiversity analyses.

Biodiversity was estimated through different alpha and beta indexes. Menhinick's index (I_{Mn}) was used to evaluate species richness. This index is based on the presumed linear relationship between the species richness and the total number of individuals. The Shannon–Weaver index (H') was used to estimate diversity and reflected the amount of disorder in the species distribution of the observed community. Evenness, or equitability, was measured through Pielou's index (J'). This index provided a sense of how evenly the different species contributed to the Shannon–Weaver diversity index. Simpson's reciprocal index ($1/D$) measured the number of equally common species that will produce an observed Simpson's index (D), which measures dominance. These alpha indexes were used to display the changes in the communities during fermentation, allowing also comparisons among them. They were calculated according to the following equations (Magurran, 2004):

$$I_{Mn} = S/\sqrt{N} \quad (1)$$

$$H' = -\sum p_i * \ln(p_i) \quad (2)$$

$$J' = H'/\ln S \quad (3)$$

$$1/D = 1/\sum p_i^2 \quad (4)$$

where p_i is the relative abundance of species i, S is the number of species present and N is the total number of individuals. Beta indexes were used to evaluate pairwise similarities between whole microbial communities, which were determined by calculating Jaccard's similarity coefficient (*Sj*) and Whittaker's index of association (*Sw*) (Whittaker, 1952) using the following equations (Legendre and Legendre, 1998):

$$Sj = W/(a1 + a2 - W) \quad (5)$$

where W is the number of species shared between populations 1 and 2, while $a1$ and $a2$ are the total number of different species in populations 1 and 2, respectively;

$$Sw = 1 - \sum |b_i1 - b_i2|/2 \quad (6)$$

where b1 and b2 are the percentage contributions of the ith species in samples 1 and 2, respectively. Both Jaccard (presence-absence) and Whittaker (proportional) indexes are measures of the similarity between communities (*patios*), with values from 0 (completely different) to 1 (identical). These indexes were used to compare changes in communities over time and between communities at each fermentation stage. Diversity indexes were calculated manually. Mean values of alpha diversity indexes among time periods were compared through the ANOVA of repeated measures in each community. Comparisons of mean values of alpha diversity indexes between communities were done by t-Student's test. Bartlett and Levene tests were used to check for homogeneity of the variance, while Kolmogorov-Smirnov test was used to check for normality. When it was necessary, values were transformed before the parametric test was carried out. To estimate diversity conservatively, singletons (species represented by just one individual) as well as unidentified microorganisms were removed prior to community analyses, as suggested by Zhou *et al*. (2013).

3. Results

3.1 Physico-chemical analyses.

NaCl concentration in the brines reached an equilibrium during the first week of fermentation, showing values of 7.76 (±0.24) and 5.88 (±0.29) % (w/v) in the fermenters at *patio* #1 and #2, respectively. Values of pH evolved in a different manner in both *patios*, for in *patio* #1 brines were acidified since the beginning. In *patio* #1, pH values were 5.7 (±0.67), 4.0 (±0.1) and 3.91.0(±0.12), while in *patio* #2, pH values were 7.43 (±0.53), 4.3 (±0.11) and 4.29 (±0.14) at the initial, middle and final fermentation stages, respectively. Titratable acidity at the final stage was 1.14 g/L (±0.08) and 0.78 g/L (±0.08), while combined acidity was 0.14 (±0.04) and 0.16 (±0.01) Eq/L for *patio* #1 and #2, respectively. All these parameters were considered normal for this table olive preparation.

3.1. Microbiological analyses.

Averaged total counts of microorganisms isolated in the different culture media used in this study are shown in Table 2. Significant differences could be found between both *patios* in most culture media and fermentation stages. Higher count numbers were found in *patio* #2 in MRS-BPB, BHI and MacConkey in most cases. In contrast, higher counts were found in OGYE (mostly yeast) in *patio* #1 at the initial and middle fermentation stages. Nevertheless, total number of microorganisms isolated in MRS-BPB (mostly LAB) and OGYE were not significantly different at the final stage of the fermentation (Table 2). Microorganisms isolated in RCM at the middle and final stages of the fermentations were so scarce that no statistical tests could be properly carried out, although counts were very similar in both *patios* at every stage. Considering the dynamics of microbial populations along the time, significant differences in all culture media, except in OGYE, could be found in *patio* #2, while such time effect could only be detected in the evolution of microorganisms isolated in MRS-BPB and OGYE from *patio* #1.

3.1 Bacterial diversity and dynamics

Bacterial species isolated as well as the number of isolates along the Spanish-style green olive fermentation in two different *patios* are shown in Table 3, where they are arranged regarding their abundance. Also, the relative abundance of bacterial species found in each of the 20 fermenters under study, at the three fermentation stages considered, is shown in Fig. 1. A total of 37 different species were found, belonging to 18 different bacterial genera. The vast majority were Gram positive bacteria, i.e. 76% and 80% in *patio* #1 and #2, respectively. It was noteworthy the ubiquitous presence of the species *L. pentosus* in all 20 fermenters under study at virtually every sampling point (Fig. 1). Seven other species could be also found in both *patios*, i.e. *Lactobacillus paracollinoides/collinoides*, *Lactobacillus parafarraginis*, *Lactobacillus rapi*, *Pediococcus ethanolidurans*, *Staphilococcus* sp., *Pediococcus parvulus* and *Paenibacillus illinoisensis/xylanilyticus* (Table 3). In addition, all these species, except *P. illinoisensis/xylanilyticus*, were isolated at the same fermentation stages from both *patios*, and especially at the final stage (Table 3). With up to16 species found, it is remarkable the prevalence of LAB in both *patios*: *ca*. 92% and 97% of the isolates in *patio* #1 and #2, respectively, and 72% and 93%, respectively, when removing the *L. pentosus* isolates. The maximum number of distinct species was found at the initial stage of fermentation, so that 22 out of the 37 bacterial species found were isolated only at this occasion, 13 of them from *patio* #1 and 8 of them from *patio* #2, being *L. pentosus* the only common species at this stage. Nevertheless, many of the species which were only isolated at the first stage could only be detected in one or two of the fermenters in each *patio*. The exceptions were the species *Enterococcus casseliflavus*, *Vibrio furnissii/fluvialis* and *Weisella paramesenteroides/hellenica* in *patio* #1, and *Aerococcus viridans/urinaeequi* and *Enterococcus saccharolyticus* in *patio* #2, which were isolated from most fermenters at each *patio* (Table 3). In contrast, a few species could be detected only at the final stage of fermentation: *Pantoea agglomerans* in *patio* #1, and *L. paracollinoides/collinoides*, *Pediococcus ethanolidurans* and *L. parafarraginis* in *patio* #2. While only *L. pentosus* could be isolated from all of the fermenters in *patio* #1, *P. parvulus* and *E. saccharolyticus*, apart from *L. pentosus*, were isolated from all fermenters in *patio* #2. Other species which were isolated from 6 or more fermenters at each *patio* were *L. paracollinoides/colinoides*, *L. parafarraginis*, *V. furnisii/fluvialis*, *Staphilococcus* sp. and *W. paramesenteroides/hellenica* in *patio* #1, and *A. viridans/urinaeequi* and *L. paracasei* in *patio* #2.

Regarding the counts of each species, with the exception of *A. viridans/urinaeequi* and *E. saccharolyticus* in *patio* #2, those which reached the largest concentrations in the brines (more than 10^5 CFU/ml) were isolated at the middle and final stages of fermentation (Table 3). Again, the prevalence of the species *L. pentosus* was clear but other species reached high count numbers. This was especially true for most LAB species (lactobacilli, pediococci and enterococci), but also for *Staphylococcus* sp. (Table 3). Species belonging to the enterobacteriaceae group were all isolated only at the initial stage, except for *P. agglomerans* at the final stage, being their counts as well as the number of fermenters colonised by this group extremely low.

Authors that, to our knowledge, have cited the isolation or DNA amplification of any of the bacterial species found in this study, either in Spanish-style or any other table olive preparations, are referenced in Table 3. A total of 20 bacterial species, i.e. more than 50%, have been isolated for the first time from Spanish-style green olive fermentations in this study, while 17 had not been described before in any table olive preparation. The genera *Brachybacterium, Paenibacillus, Sporolactobacillus, Paracoccus* and *Yersinia* had not been cited before from any table olive preparation to our knowledge.

3.2 Yeast diversity and dynamics

Yeast species isolated along the Spanish-style green olive fermentation in the two *patios* of this study, arranged according to their abundance, are shown in Table 4. The relative abundance of yeast species found in each of the 20 fermenters under study, at the three fermentation stages considered, is shown in Fig. 2. Taking into account that 24 isolates from *patio* #1 could not be assigned to any specific species with a minimum of confidence, a total of 12 different species were found, belonging to 7 different yeast genera. More yeast isolates and species diversity was found in *patio* #1 than in *patio* #2, especially at the initial fermentation stage (Table 4). Three yeast species could be isolated from both *patios*, i.e. *Saccharomyces cerevisiae, Candida thaimueangensis* and *Hanseniaspora sp.*, being also detected at similar fermentation stages. In contrast with the results obtained for bacteria, two yeast species appeared to somehow dominate the yeast microbiota: *C. thaimueangensis* and *S. cerevisiae* (Table 4). These two species were isolated from most fermenters in both *patios* at most fermentation stages. *S. cerevisiae* appeared to be dominant at the initial and middle stages, while *C. thaimueangensis* increased its presence as fermentation progressed and dominated the

final stage. Regarding their relative abundance, counts were especially high for *Saccharomyces* sp. (Table 4). *Issatchenkia orientalis* and different species of *Candida* were also very abundant in most fermenters of *patio* #1, where they were isolated mostly at the first fermentation stage (Table 4). As for bacteria, authors that have cited the isolation or DNA amplification of any of the yeast species found in this study are referenced in Table 4. To our knowledge, the species *Candida butyri/asseri* and *Rhodotorula mucilaginosa* had not been described before from Spanish-style green olive fermentations, while the species *Saturnispora mendoncae* had not been cited before from any table olive preparation (Table 4).

3.3 Biodiversity analyses.

3.3.1 Alpha diversity indexes.

Total bacterial species richness found was identical in each *patio* once singletons were removed, i.e. 15 species (Table 3), although this figure was lower when looking at each of the three fermentation stages considered (Table 3) or a particular fermenter (Fig. 1). Species richness was evaluated through the Menhinick's diversity index (I_{Mn}), which is shown in Fig. 3 (panel A). This index showed a decrease in its values as fermentation progressed in *patio* #1, with statistically significant differences between the initial and final stages of fermentation (Fig. 3). No significative differences, though, were found in *patio* #2 or between both *patios* at any fermentation stage. Bacterial diversity, evaluated by the Shannon-Weaver index (*H'*), is shown in Fig. 3 (panel B). Although the values of this index became lower as fermentation proceeded, no significative difference was found neither through the fermentation stages in any *patio* nor between both *patios*. Maximum values were always reached at the initial stage. The decrease in the values of the *H'* index in *patio* #2 can be explained by a parallel decrease of evenness in the distribution of the species found, as indicated by the Pielou's index (*J'*) (Fig. 3, panel C). Actually, the difference of evenness is statistically significant between the initial and final stages of fermentation in *patio* #2. Dominance, as expressed by Simpson's reciprocal index (1/*D*) (Fig. 3, panel D), followed a pattern similar to bacterial diversity evaluated trough the Shannon-Weaver index (Fig. 3, panel B). Again, no significant difference was found between both *patios* at any stage of the fermentation. However, a significant difference could be found between the initial and final stages in *patio* #2. This is due to the fact that Simpson's reciprocal index put more weight on most abundant species, being more influenced by the values of evenness indexes than those

of species richness. This explains the fact that, although there is an increase in species richness in *patio* #2 along fermentation time, these species are less evenly distributed, producing a statistically significant change in the nature of dominant species. In contrast, in *patio* #1 evenness is quite similar across the three fermentation stages so that the decrease in the values of Simpson's reciprocal index is again due to loss of species richness.

Yeast species richness was quite lower than bacterial one, being also quite different in the two *patios* under study (Table 4). The low number of species isolated when sampling any fermenter along the fermentation time made advisable to calculate diversity indexes globally for each fermenter, i.e. not considering the fermentation stages. The values of these indexes are shown in Fig. 4. Significative differences between both *patios* were found in the values for the Shannon-Weaver's (*H'*), Pielou's (*J'*) and Simpson's reciprocal (*1/D*) indexes, being these values always higher in *patio* #1 (Fig. 4). However, no significant difference was found regarding species richness estimated through Menhinick's index (I_{Mn}). This is the result of an unequal amount of sampling effort in both patios, for yeast counts on OGYE medium were significantly lower (Table 2) and its species composition less diverse (Table 4) in *patio* #2 at the initial and middle fermentation stages.

3.3.2 Beta diversity indexes.

Pair-wise comparisons of microbial community composition using Jaccard and Whittaker beta diversity indexes for bacteria and yeast are shown in Table 5. Regarding bacteria, the similarity between both communities, i.e. *patio* #1 and #2, became higher as fermentation went on from the initial to the final fermentation stages. Values obtained for Jaccard's coefficient were always lower than those for Whittaker's index, indicating that species shared by both *patios* were also the most abundant. This was supported by the fact that the species *L. pentosus* actually dominated all along the fermentation in both *patios* (Table 3 and Fig. 1). In addition, Jaccard's coefficient allowed us to perceive the ecological succession in the species structure of each community (*patio*) over time. Changes in the species composition were gradual, being more similar this composition at the middle and final fermentation stages. Finally, Whittaker's index values were higher for *patio* #1 than for *patio* #2, a result that is a consequence of a change in the species distribution in *patio* #2 between the initial and middle stages of the fermentation. More specifically, *L. pentosus* is the co-dominant

461 species together with *A. viridans/urinaeequi* during the initial stage of fermentation in
462 this *patio*, while *L. pentosus* alone is the dominant species for the rest of the
463 fermentation (Table 3 and Fig. 1).
464 In contrast to the results showed by the bacterial community, maximum
465 similarity for yeast community composition between both *patios* was found at the
466 middle stage of fermentation (Table 5). As for bacteria, an ecological succession was
467 also observed over time, with a species composition more similar at the middle and final
468 stages of fermentation. In the case of *patio* #2 the change in the yeast species
469 composition is complete between the initial and final stages, as denoted by the 0.00
470 value for both Jaccard's and Whittaker's indexes (Table 5).
471
472
473 **4. Discussion**
474
475 The aim of this study was to update our knowledge on the microbiota associated
476 to table-olive fermentations produced through the Spanish-style procedure. To
477 accomplish this task we have used both, classic microbiological (culture dependent)
478 techniques and modern molecular techniques for the identification of the different
479 bacterial and yeast species isolated. In addition, our goal was not only to describe this
480 microbiota and its evolution (dynamics) along the olive fermentations, but also to
481 recover the microbial diversity associated to this traditional food fermentation as well as
482 to preserve it for further biotechnological purposes. For this reason, our sampling
483 strategy included the recovery of all morphological types appearing in the different
484 culture media used, instead of the more usual "random" picking of the isolated colonies.
485 In this sense, it was of the greatest value the use of a modified MRS-agar culture
486 medium which included bromophenol blue as a discriminating agent of the actual
487 metabolism/morphology of the isolates growing onto this medium, as proposed by Lee
488 and Lee (2008)(see an example in the supplementary Fig. S1).
489 Spanish-style green olive fermentations appeared to be dominated by the species
490 *L. pentosus*. This observation is not novel, for other authors have reported this fact
491 previously (de Castro et al., 2002; Ruiz-Barba and Jiménez-Díaz, 2012; Hurtado et al.,
492 2012; Heperkan 2013, among others). Furthermore, it is remarkable the ubiquitous
493 presence of this species in all fermenters, at medium-high counts, since very early at the
494 first stage of the fermentation. Therefore, it is not surprising that 68% of the total

isolates belonged to this species. Apart from *L. pentosus*, a remarkably high number of LAB species, 15 in total, were isolated. Some of these species had not been described before from Spanish-style table-olive fermentations, i.e. *W. paramesenteroides/hellenica, P. parvulus, E. saccharolyticus, L. rhamnosus* and *S. inulinus/terrae*. Actually, two of these species, i.e. *E. saccharolyticus* and *S. inulinus/terrae*, had not been cited before from any table-olive preparation. As many of these LAB species have been described as exerting some probiotic effect (Fontana *et al*., 2013), Spanish-style olive fermentation brines were revealed as a valuable source of potentially probiotic strains. In addition, *Enterococcus* species appeared to have a role at the crucial initial stage, with *E. casseliflavus* and *E. saccharolyticus* in *patio* #1 and #2, respectively. This observation was not novel, for actually De Castro et al. (2002) described the use of *E. casseliflavus* and *L. pentosus* as mixed starter cultures for Spanish-style green olive fermentation. Such use was based on the high-pH tolerance of *Enterococcus* species as well as its LAB character. Finally, two quite abundant and ubiquitous bacterial species were isolated at the initial stage of the fermentation whose 16S DNA showed similarity to the species *Vibrio furnisii/fluvialis* and *E. saccharolyticus*, respectively. However, the percentages of similarity ($\leq$ 97%) of the 16S DNA amplicon studied here, as well as other phenotypic (sugar metabolism) and genetic characteristics (DNA-DNA similarity) which have been investigated so far, suggested that these could constitute two novel species. Current efforts in our laboratory are focused on this purpose.

Yeast species were less abundant than bacteria, both in counts and number of species. Two yeast species appeared to be inherent to the Spanish-style green olive fermentation in both *patios*, i.e. *S. cerevisiae* and *C. thaimueangensis*. In a recent study on the yeast diversity of table-olive fermentations, Bautista-Gallego et al. (2011) described the species *Candida tropicalis* and *Pichia galeiformis* as dominant in Spanish-style Manzanilla-variety olive fermentations in a manufacturing company which is, actually, geographically quite close to *patio* #1 studied here. Although these authors did not find *S. cerevisiae*, they described the isolation of *C. thaimueangensis*, but restricted just to the final fermentation stage while obtaining low number of isolates (12% of the yeast isolates at that stage). As for bacteria, it is very interesting to find yeast species not cited before either in Spanish-style, i.e. *C. butyri/asseri* and *R. mucilaginosa*, or in any table-olive preparation, as it is the case with *S. mendoncae*. This

fact reinforces the idea of table-olive brines as a source of novel yeast strains with desirable biotechnological properties.

After evaluating microbial diversity through different alpha and beta indexes, our results showed again that this food fermentation was dominated by a single species, i.e. *L. pentosus*. Therefore, it was not unexpected that the actual values of different diversity indexes were relatively low, especially when singletons were removed from the analyses. For bacteria, although not always significative differences could be found, maximum diversity was displayed at the initial fermentation stage. Evenness, i.e. the frequency distribution of the different species, also decreased along the fermentation. This was due to the dominance exerted by the species *L. pentosus*, although statistically significant differences could only be observed in *patio* #2. This effect was most probably due to the change in the dominant species from the initial stage, i.e. *A. viridans/urinaeequi*, to the middle and final stages, dominated by *L. pentosus*. No significant differences could be found in any diversity index between both *patios* at any fermentation stage, suggesting that the process is quite "robust" once properly started. Although diversity was very similar in both *patios*, differences could be found in the actual composition of the "accessory" microbiota, i.e. that accompanying *L. pentosus* species. Nevertheless, most of this "accessory" microbiota was composed of other LAB and could represent a sort of "watermark" of a particular *patio*. Similar studies on consecutive olive fermenting seasons at the same *patios* could prove or discard such a hypothesis. On the other hand, yeast diversity was much lower than bacterial one, with significative differences between both *patios*. Diversity, evenness and dominance indexes were all higher in *patio* #1. This fact did not appear to have an effect on the outcome of the fermentation, estimated through the physical and chemical analyses used in this study. As other authors have described different yeast species compositions (Bautista-Gallego et al., 2011), especially regarding the dominant species, no critical role could be predicted for this microbial group in Spanish-style olive fermentation apart from its not-yet demonstrated, but suggested, influence on the organoleptic properties of the product (Arroyo-López et al., 2008).

We believe that this microbiological study is quite representative of the Spanish-style green olive fermentation because of the selection of two large, well-established and traditional table-olive manufacturing companies in the geographical area of maximum world production. In addition, the number and capacity of the fermenters from which samples were obtained, twenty 10-tonne fermenters representing *ca.* 200

tonnes of fermenting table olives, contributed to consistent and comprehensive results which will no doubt update our knowledge on this important food fermentation.

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Legends to Figures.

Figure 1. Bacterial species frequency in ten fermenters of the fermentation yard (*patio*) # 1 (panel A) and #2 (panel B). For each fermenter, from left to right, the three bars represent the bacterial species frequency at the initial, middle and late stages of fermentation, respectively.

Figure 2. Yeast species frequency in ten fermenters of the fermentation yard (*patio*) # 1 (panel A) and #2 (panel B). For each fermenter, from left to right, the three bars represent the yeast species frequency at the initial, middle and late stages of fermentation, respectively.

Figure 3. Diversity indexes for bacteria in two fermentation yards (*patios*) along the three (initial, middle and final) stages of Spanish-style green olive fermentation. Panel A: Menhinick's diversity index (I_{Mn}); panel B: Shannon-Weaver's diversity index (H'); panel C: Pielou's evenness index (J'); panel D: Simpson's reciprocal index (1/D). Ten fermenters were studied at each *patio* (n=10); bars indicate standard errors; * indicates a significant difference ($p<0.05$). Singleton species have been removed from the analyses.

Figure 4. Diversity indexes for yeast in two fermentation yards (*patios*) processing Spanish-style green olives. Panel A: Menhinick's diversity index (I_{Mn}); panel B: Shannon-Weaver's diversity index (H'); panel C: Pielou's evenness index (J'); panel D: Simpson's reciprocal index (1/D). Ten fermenters were studied at each *patio* (n=10); bars indicate standard errors; * indicates a significant difference ($p<0.05$). Singleton species and unidentified yeast have been removed from the analyses.

Supplementary material - Figure Legend

Figure S1. Colonies of bacteria growing onto MRS-BPB, a modified MRS-agar which included bromophenol blue (Lee and Lee, 2008) as a discriminating agent of the actual metabolism of the isolates.

Table 1. Primers used in this study.

Primer	Sequence (5'- 3')	Reference
OPL5	ACGCAGGCAC	Maldonado-Barragán et al., 2013
ISS1rev	GGATCCAAGACAACGTTTCAAA	Veyrat et al., 1999
plb16	AGAGTTTGATCCTGGCTCAG	Kullen et al., 2000
mlb16	GGCTGCTGGCACGTAGTTAG	Kullen et al., 2000
paraF	GTCACAGGCATTACGAAAAC	Torriani et al., 2001
pentF	CAGTGGCGCGGTTGATATC	Torriani et al., 2001
planF	CCGTTTATGCGGAACACCTA	Torriani et al., 2001
pREV	TCGGGATTACCAAACATCAC	Torriani et al., 2001
PAR	GACGGTTAAGATTGGTGAC	Ventura et al., 2003
CAS	ACTGAAGGCGACAAGGA	Ventura et al., 2003
RHA	GCGTCAGGTTGGTGTTG	Ventura et al., 2003
CPR	CAANTGGATNGAACCTGGCTTT	Ventura et al., 2003
NL1	GCATATCAATAAGCGGAGGAAAAG	Kurtzman and Robnett, 1998
NL4	GGTCCGTGTTTCAAGACGG	Kurtzman and Robnett, 1998

Table 2. Averaged microbial counts along Spanish-style green-olive fermentations in two fermentation yards (*patios*) obtained in the culture media used in this study.

Culture medium	Fermentation stage	Patio 1	Patio 2	P-value[1]
MRS-BPB	Initial	3.78 (0.74)[2]	6.73 (0.69)	0.000
	Middle	5.78 (0.84)	7.33(0.23)	0.000
	Final	6.19(0.79)	6.42(0.20)	NS[4]
Sig.[3]		*	*	
BHI	Initial	4.42(0.91)	6.60(0.70)	0.000
	Middle	5.34 (0.89)	7.38 (0.35)	0.000
	Final	5.46(0.82)	6.37(0.30)	0.006
Sig.			*	
MacConkey	Initial	4.18(1.27)	2.06(1.99)	0.015
	Middle	4.68(1.26)	6.38 (0.44)	0.001
	Final	3.29(2.12)	5.47(0.41)	0.013
Sig.			*	
RCM	Initial	1.24(0.86)	1.19(1.12)	NS
	Middle	0.51(0.82)	0.37(0.78)	-[5]
	Final	0.34(1.08)	0.17(0.54)	-
Sig.		-	-	
OGYE	Initial	3.83 (0.43)	2.47(1.25)	0.010
	Middle	3.56(1,19)	2.10(1.69)	0.045
	Final	2.62(1.04)	2.74(0.52)	NS
Sig.		*		

[1]Statistical significance considering both *patios* at each fermentation stage (U Mann-Whitney's test; for P ≤0.05). [2]Mean log CFU/ml (standard deviation), n=10. [3]Sig.: statistical significance of time effect in the fermentation within each *patio* (Friedman-test; * for P<0,05). [4]NS, not significant difference. [5]-, not enough data to carry out the statistical test.

Table 4. Yeast species isolated along Spanish-style green olive fermentations in two different fermentation yards ("*patios*").

Patio 1	Fermentation stage			Total [1]	No.	Count range[3]	References[4]	
Yeast species	initial	middle	final	isolates	Ferm.[2]	(log CFU/ml)	Spanish-style	Other
Saccharomyces cerevisiae	2[5]	12	3	17	9	1-5	a-e	e-q
Issatchenkia orientalis	17	0	0	17	8	1-2	a-c,m, r	n, q, s, t
Candida tropicalis	12	0	0	12	6	1-2	a, c, d, m	m
Candida thaimueangensis	1	4	7	10	7	1-2	m	m
Candida butyri/aaseri	9	0	0	9	6	1-2		j, n, u
Rhodotorula mucilaginosa	0	0	4	4	4	1		j,n,p,v,w
Saturnispora mendoncae	3	0	0	3	3	2		
Hanseniaspora sp.[6]	3	0	0	3	3	1-2	m	g, l
*Candida parapsilosis**	0	0	1	1	1	1	a, b, d, r	h, i, u
Other yeast sp.[7]	21	3	0	24	9	1-4		
Total isolates[8]	68	19	15	102[9]				
Species richness	8	3	4	10				
Species richness w/o singletons	8	3	3	9				

Patio 2	Fermentation stage			Total [1]	No.	Count range[3]	References[4]	
Yeast species	initial	middle	final	isolates	Ferm.[2]	(log CFU/ml)	Spanish-style	Other
Candida thaimueangensis	0	2	17	19	10	1-2	m	m
Saccharomyces cerevisiae	8	2	0	10	8	1-4	a-e	e-q
Kluyveromyces lactis/marxianus	0	4	0	4	4	1	m	i, u
Pichia manshurica/membranifaciens	0	1	3	4	4	1	a-d, m	j-o,q,s,t,w-z
Hanseniaspora sp.[6]	1	0	0	1	1	1-4	m	g, l
*Candida glabrata**	1	0	0	1	1	1	a, c	i, v
Total isolates[8]	10	9	20	39[9]				
Species richness	3	4	2	6				
Species richness w/o singletons	2	4	2	5				

[1]Total isolates of a specific yeast species; [2]Number of fermentors, out of a total of ten, from which a specific yeast species was isolated in each *Patio*; [3]Colony count range at which that yeast species was isolated; [4] Bibliographic reference which cited that particular yeast species in Spanish-style and/or other table olive preparations; [5] Number of isolates of that yeast species at that sample point; [6]The most homologous species were *Hanseniaspora opuntiae*, *H. meyeri*, *H. lachancei* and *H. uvarum*; [7]These yeast isolates could not be adscribed to any specific yeast species; [8] Total yeast isolates at each sampling point; [9]Total yeast isolates in each *Patio*. Key to references: a, González-Cancho, F. 1963; b, González-Cancho, F. 1965; c, González-Cancho, F. 1966a; d, González-Cancho, F. 1966b; e, Garrido-Fernández *et al.*, 1997; f, Marquina *et al.*, 1992; g, Arroyo-López *et al.*, 2006; h, Mourad and Nour-Eddine, 2006; i, Hernández *et al.*, 2007; j, Nisiotou *et al.*, 2010; k, Rodríguez-Gómez *et al.*, 2010; l, Silva *et al.*, 2011; m, Bautista-Gallego *et al.*, 2011; n, Muccilli *et al.*, 2011; o, Abriouel *et al.*, 2011; p, Alves *et al.*, 2012;q, Golomb *et al.*, 2013;r, Mrak *et al.*, 1956;s, González-Cancho *et al.*, 1975;t, Doulgeraki *et al.*, 2012;u, Hurtado *et al.*, 2008;v, Campaniello *et al.*, 2005;w, Franzetti *et al.*, 2011;x, Oliveira *et al.*, 2004; y, Coton *et al.*, 2006; z, Chamkha *et al.*, 2008. *Species which have been considered singletons and have been removed from the diversity analyses.

Table 5. Pair-wise comparisons of microbial community composition values in Spanish-style green olive fermentations using Jaccard and Whittaker beta diversity indexes.

		Beta diversity indexes			
Pair-wise comparisons		Bacteria		Yeast	
Patio	Fermentation stage	*Sj*[a]	*Sw*[b]	*Sj*	*Sw*
1	Initial/Middle	0.25	0.67	0.29	0.06
1	Middle/Final	0.50	0.88	0.67	0.46
1	Initial/Final	0.14	0.66	0.25	0.06
2	Initial/Middle	0.08	0.39	0.20	0.22
2	Middle/Final	0.64	0.86	0.50	0.33
2	Initial/Final	0.07	0.39	0.00	0.00
1/2[c]	Initial	0.08	0.39	0.29	0.11
1/2	Middle	0.15	0.71	0.50	0.44
1/2	Final	0.64	0.81	0.25	0.50

[a]Jaccard's coefficient; [b]Whittaker's index of association; [c]Comparison of the community composition between both patios at the different fermentation stages.

Table 3. Bacterial species isolated along Spanish-style green olive fermentations in two different fermentation yards ("*patios*").

Patio 1	Fermentation stage			Total [1]	No. [2]	Count range[3]	References[4]	
Bacterial species	initial	middle	final	isolates	Ferm.	(log CFU/ml)	Spanish-style	Other
Lactobacillus pentosus	74[5]	98	135	307	10	1-6	a - f	d, e, g - n
Lactobacillus paracollinoides/collinoides	0	20	13	33	8	1-5	f	i, l, o
Pediococcus ethanolidurans	0	3	18	21	4	1-5	f	k
Enterococcus casseliflavus	11	0	0	11	5	1-2	a	h
Lactobacillus parafarraginis	0	4	6	10	7	1-5	f	
Vibrio furnissii/fluvialis[6]	9	0	0	9	6	2-3		
Staphylococcus sp.[7]	3	2	2	7	6	1-5		
Weissella paramesenteroides/hellenica	7	0	0	7	6	1		h
Lactobacillus plantarum	5	0	0	5	2	1-3	d, e, p - r	d,g,h,k,l,n,o,s,t
Pediococcus parvulus	0	0	4	4	2	3-5		j, l, m
Clostridium xylanovorans	3	1	0	4	4	1		
Propionibacterium acnes	0	0	3	3	1	4	u	
Escherichia sp.[8]	2	0	0	2	1	1	v, w	
Lactobacillus rapi	0	0	1	1	1	3	f	
*Pantoea agglomerans**	0	0	1	1	1	3		i
*Bacillus circulans**	1	0	0	1	1	1		
*Bacillus weihenstephanensis/mycoides**	0	1	0	1	1	1		
*Brachybacterium muris**	0	1	0	1	1	1		
*Clostridium jejuense**	1	0	0	1	1	1		
*Clostridium sartagoforme**	1	0	0	1	1	1		
*Clostridium schirmacherense/argentinense**	1	0	0	1	1	1	x	x
*Enterobacter hormaechei**	1	0	0	1	1	1		
*Enterobacter radicincitas/oryzae**	1	0	0	1	1	1		
Enterobacter sp[9] *	1	0	0	1	1	1	v, w, y	
Paenibacillus illinoisensis/xylanilyticus	0	1	0	1	1	1		
Total isolates[10]	121	131	183	435[11]				
Species richness	15	9	9	25				
Species richhness w/o singletons	8	7	8	15				

Patio 2	Fermentation stage			Total[1]	No. [2]	Count range[3]	References[4]	
Bacterial species	initial	middle	final	isolates	Ferm.	(log CFU/ml)	Spanish-style	Other
Lactobacillus pentosus	48[5]	109	168	325	10	1-7	a - f	d, e, g - n
Aerococcus viridans/urinaeequi	55	0	0	55	9	1-5	z	
Pediococcus parvulus	0	15	19	34	10	4-6		j, l, m
Lactobacillus paracasei	0	18	2	20	7	3-7	d	h, l, n, s, t
Enterococcus saccharolyticus[6]	16	0	0	16	10	2-6		
Lactobacillus coryniformis	0	4	6	10	5	4-6	b	h, k, l
Lactobacillus rhamnosus	0	2	4	6	3	4-5		h, s, t
Staphylococcus sp.[7]	0	1	5	6	5	1-5		
Lactobacillus rapi	0	3	2	5	4	4-6	f	
Lactobacillus paracollinoides/collinoides	0	0	4	4	4	4-5	f	i, l, o
Pediococcus ethanolidurans	0	0	2	2	1	4	f	k
Paenibacillus sp.[12]	2	0	0	2	2	1		
Sporolactobacillus inulinus/terrae	0	2	0	2	1	1		
Lactobacillus parafarraginis	0	0	1	1	1	4	f	
*Lactobacillus paraplantarum**	1	0	0	1	1	3	e	d, g, k, n
*Enterobacter kobei**	1	0	0	1	1	2		
*Escherichia coli**	1	0	0	1	1	2	v, w	
*Paracoccus carotinifaciens**	1	0	0	1	1	2		
Paenibacillus illinoisensis/xylanilyticus	1	0	0	1	1	1		
*Yersinia enterocolitica**	1	0	0	1	1	1		
Total isolates[10]	127	154	213	494[11]				
Species richness	10	8	10	20				
Species richness w/o singletons	5	8	10	15				

[1]Total isolates of a specific bacterial species; [2]Number of fermentors, out of a total of ten, from which a specific bacterial species was isolated in each *patio*; [3]Colony count range at which that bacterial species was isolated; [4] Bibliographic reference which cited that particular bacterial species in Spanish-style and/or other table olive preparations; [5]Number of isolates of that bacterial species at that sample point; [6]The relatively low (≤97%) 16S rDNA homology of these isolates with other bacterial species in the data banks could indicate that they might be novel species; [7]The most homologous species were *Staphilococcus epidermidis, S. saccharoliticus, S. capitis* and *S. caprae*; [8]The most homologous species were *Escherichia coli, E. senegalensis* and *E. fergusonii*; [9]The most homologous species were *Enterobacter cloacae, E. sacchari, E. kobei* and *E. radicincitas*; [10]Total bacterial isolates at each sampling point; [11]Total bacterial isolates in each *patio*; [12]The most homologous species were *Paenibacillus taichungensis, P.tundrae, P. tylopili,* and *P. barcinonensis, P.amylolyticus.* Key to references: a, De Castro *et al.*, 2002; b, Aponte *et al.*, 2012; c, Ruiz-Barba and Jiménez-Díaz, 2012; d, Doulgeraki *et al.*, 2013; e, Bautista-Gallego *et al.*, 2013; f, Montaño *et al.*, 2013; g, Hurtado *et al.*, 2008; h, De Bellis *et al.*, 2010; i, Abriouel *et al.*, 2011; j, Franzetti *et al.*, 2011; k, Doulgeraki *et al.*, 2012; l, Randazzo *et al.*, 2012;m, Abriouel *et al.*, 2012; n, Argyri *et al.*, 2013; o, Chamkha *et al.*, 2008;p, Ruiz-Barba *et al.*, 1991;q, Ruiz-Barba and Jiménez-Díaz, 1994;r, Ruiz-Barba and Jiménez-Díaz, 1995;s, Balloni *et al.*, 1973; t, Mourad and Nour-Eddine, 2006; u, González-Cancho F. *et al.*, 1980; v, Borbolla y Alcalá et al., 1960;w, González-Cancho, 1963;x, Pereira *et al.*, 2008;y, Bevilacqua et al., 2010; z, González-Cancho and Durán-Quintana, 1981. *Species which have been considered singletons and have been removed from the diversity analyses.

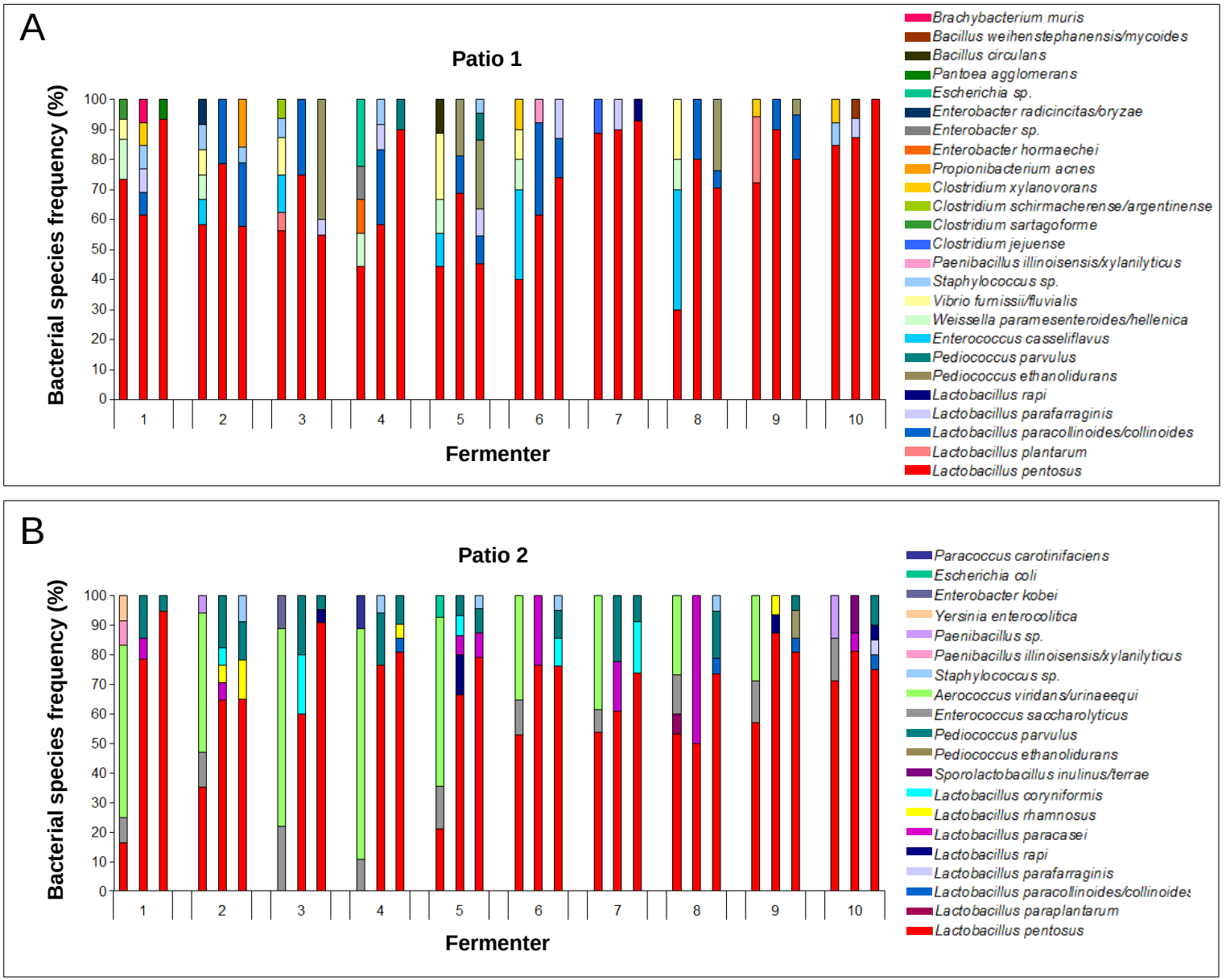

Figure 1. Helena Lucena-Padrós, Belén Caballero-Guerrero, Antonio Maldonado-Barragán and José Luis Ruiz-Barba*

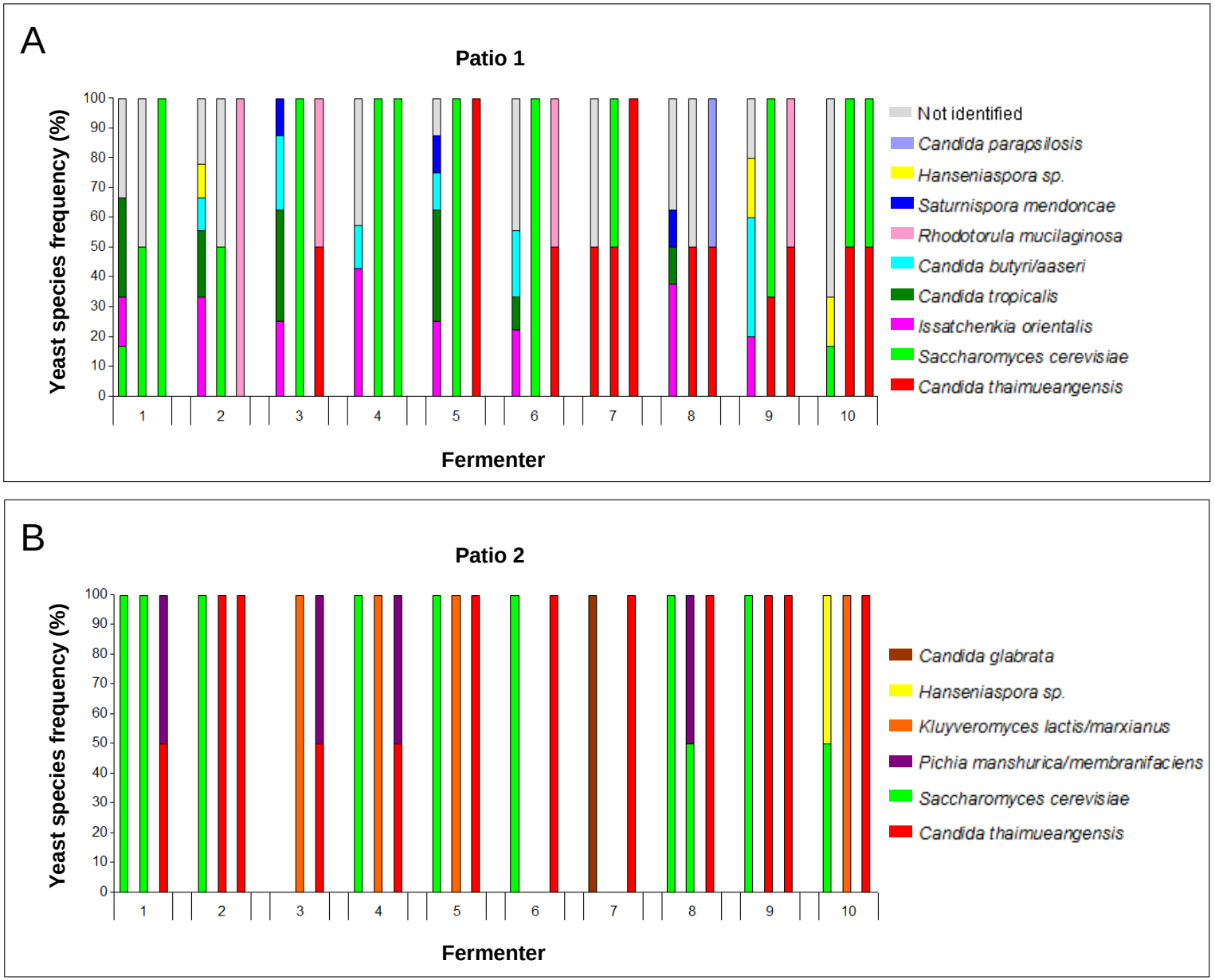

Figure 2. Helena Lucena-Padrós, Belén Caballero-Guerrero, Antonio Maldonado-Barragán and José Luis Ruiz-Barba*

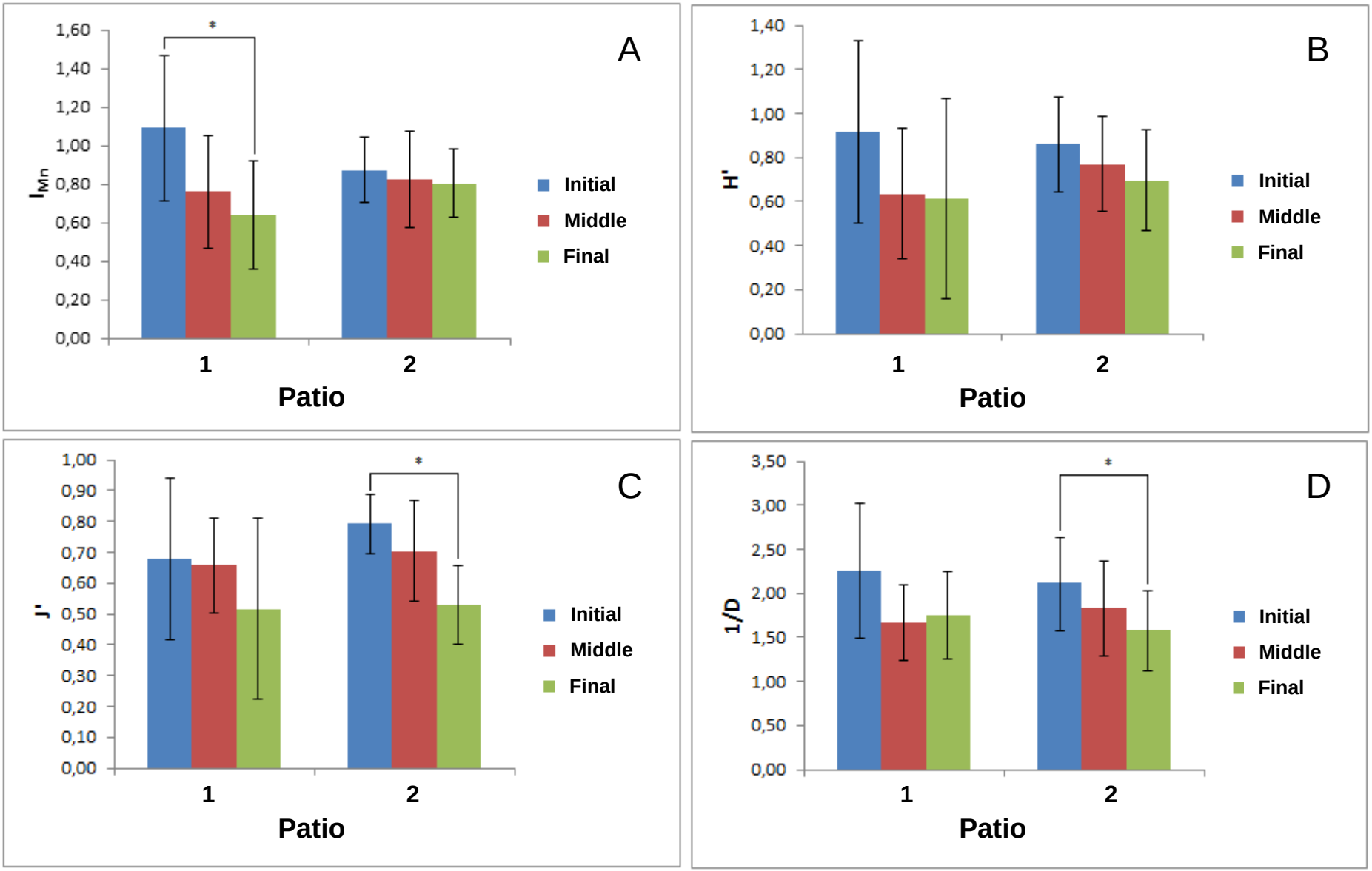

Figure 3. Helena Lucena-Padrós, Belén Caballero-Guerrero, Antonio Maldonado-Barragán and José Luis Ruiz-Barba*

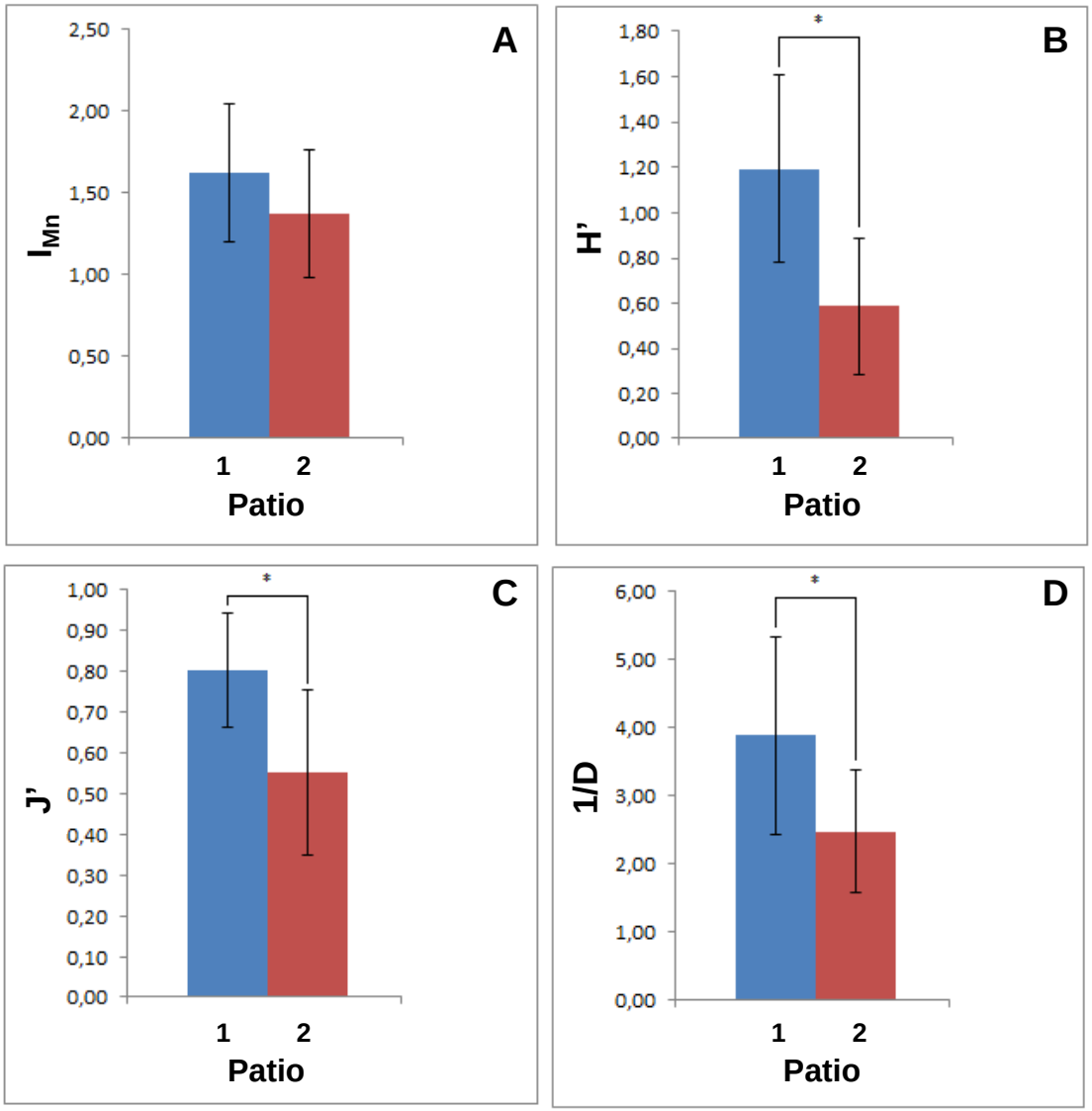

Figure 4. Helena Lucena-Padrós, Belén Caballero-Guerrero, Antonio Maldonado-Barragán and José Luis Ruiz-Barba*

Highlights

Microbial diversity and dynamics of Spanish-style green table-olive fermentations in large manufacturing companies through culture-dependent techniques.

Helena Lucena-Padrós, Belén Caballero-Guerrero, Antonio Maldonado-Barragán and José Luis Ruiz-Barba*

Highlights

- There were isolated 1070 microorganisms, 929 bacterial and 141 yeast isolates.
- Thirty-seven bacterial and 12 yeast species were isolated.
- Twenty bacterial and three yeast species novel in Spanish-style olive fermentation.
- Five bacterial and one yeast genera not cited before in table olive fermentations.
- *Lactobacillus pentosus* dominated Spanish-style olive fermentation.

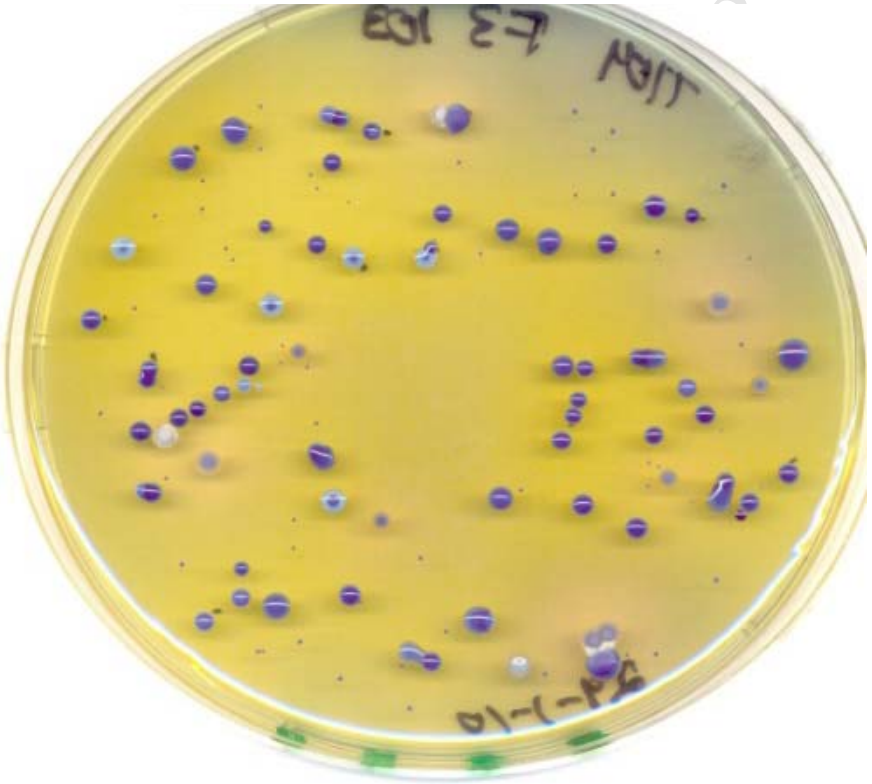

Figure S1. Helena Lucena-Padrós, Belén Caballero-Guerrero, Antonio Maldonado-Barragán and José Luis Ruiz-Barba*